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14. ABSTRACT

This final report describes the design and construction of a CGMP facility for yeast and bacteria on the first floor and basement of Othmer Hall, the home for the University of Nebraska-Lincoln Biological Process Development Facility. Total cost for construction was $11,165,740, with the University of Nebraska-Lincoln providing $5,179,226 (46%) of the funding, USAMRMC providing $4,958,049 (45%), and JVAP providing $1,028,465 (9 %). This final report also describes the expression, purification and media optimization of a monoclonal antibody against serotype A botulinum neurotoxin.

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Introduction

This project in the past has been comprised of two components. The first component, which represents 95% of the funding, is dedicated to building a Phase I/II CGMP facility in the first floor and basement of Othmer Hall, the home for the Biological Process Development Facility. The other 5% has been used to research the expression of antibodies against the botulinum neurotoxins in Chinese Hamster Ovary (CHO) cells. The botulinum antibody research is published and the manuscripts are found in the Appendix. The remainder of this final report describes the recently constructed cGMP Phase I/II Facility.

Body

Serotype A Botulinum Monoclonal Antibody

This project included the expression, purification and media optimization of the production of a monoclonal antibody against the serotype A botulinum neurotoxin. The culmination of this work was published and the manuscripts can be found in the appendix.

cGMP Construction Project

November 2006 the University of Nebraska Board Of Regents (BOR) approved a fully funded CGMP facility to be built on first floor and the basement of Othmer Hall. This CGMP facility will use a modular clean room construction for all clean room space. As of June 1, 2007 all of the bidding has been completed, contractors have been selected and demolition and construction has started. The CGMP construction team includes the following;

- AES Clean Room Technology which is responsible for design, construction, and installation of the modular clean rooms and will oversee installation of all CGMP process piping.
- BioKinetics is a subcontractor to AES and is responsible for all process and utility design, including equipment layout and process piping.
- Davis Design is a local architect responsible for all architectural elements of the project outside the scope of the modular clean rooms.
- Hampton Construction of Lincoln NE is responsible for demolition and construction of all non-clean room space, such as service utilities to the clean rooms and the support space in the basement.
- University of Nebraska-Lincoln Facility Management and Planning has oversight for the project.

The floor plan for the first floor is presented as Figure 1 and the basement is presented in Figure 2. The construction of the facility was completed December 31, 2009. Commissioning and validation of the facility, which is not a part of this project and is being funded by a different source, is scheduled to start April 2009. A description of the function of each of the spaces in the facility is presented below.

The first floor of the CGMP facility will have the following capabilities:

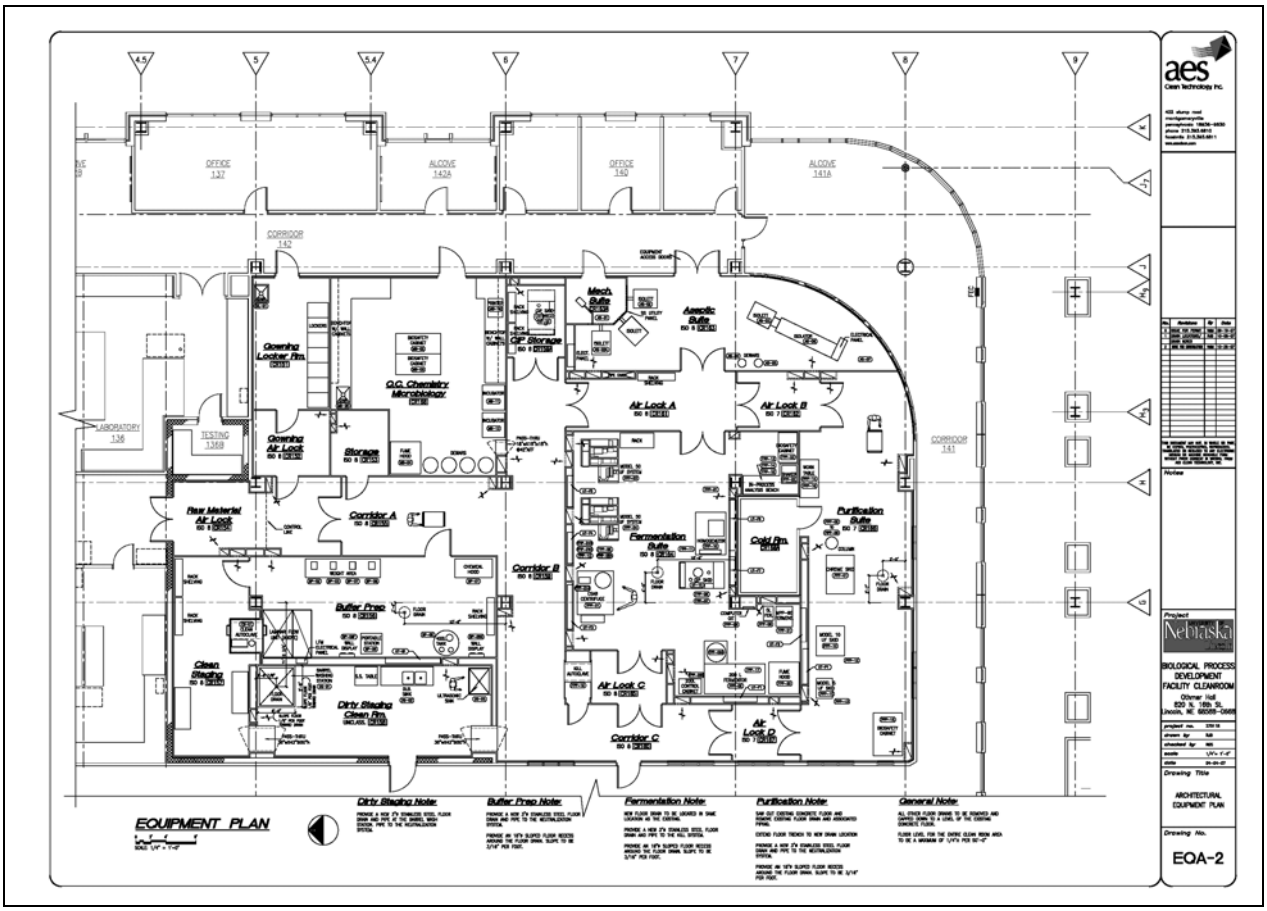
- *Fermentation Suite:* 150 L working volume fermentor for yeast and bacteria, harvesting and clarifying capabilities using either continuous centrifugation or cross flow membrane filtration and cell disruption.
- *Purification Suite:* 0.1 to 6 L/min chromatography skid and two ultrafiltration systems.
- *Asceptic Processing Suite:* Isolator system with a VHP (vaporized hydrogen peroxide) unit for sterilization capable of producing Master and Working Cell Banks and manual filling of up to 1500 liquid vials.

- *Dirty Staging.* Used for cleaning of small parts and plastic barrels that are used to hold disposable liners for buffer tanks
- *Clean staging*: Prepare items for buffer preparation or wrap and sterilize items for the processing suites.
- *Buffer Prep Area*: Prepare buffers for the processing suites.
- *Airlock and Gowning:* Changing area for cGMP facility staff.
- *Solvent Storage*: This small room is used to store methanol and ethanol that will be used in the CGMP processing suites.
- *Quality Control Laboratory*: The QC laboratory will have microbiology testing capabilities for environmental monitoring and sterility testing. The QC Laboratory will also be used for in-process testing and some final product testing. Some of the QC testing will also occur in the QC-Chemistry laboratory on the third floor of Othmer.

The Basement contains all of the support space and services to maintain the cGMP facility and include the following:

- *Raw Material Sampling Area*: This will be used to sample raw materials in a controlled area for raw material identification.
- *Raw Material Quarantine Area*: This area will be used to store released raw materials until released by QA to the Raw Material Released Area.
- *Raw Material Released Area*: This area will be used to store quarantine raw materials until released by QA to the production area.
- *Quarantine/Release Cold Room*: This cold room will be used to store raw materials that require refrigeration. The Cold Room will be segregated into a quarantine area and released area.
- *Stability Chamber Room:* The BPDF has 5 validated stability chambers with temperature ranges from -80^{o}C to 70^{o}C. This room will be used house these chambers.
- *Final Product Storage Area*: This area will house the BPDF's final product storage freezers (-80^{o}C, -20^{o}C) and refrigerators. These units are segregated into quarantine and released units.
- *Cell Bank Storage Area*: This area will be used for storing Master Cell Bank dewars and Working Cell Bank dewars.
- *Gas Storage Area*: This area is for storage of oxygen and nitrogen dewars that are need for the fermentation suite and the cell bank dewars, respectively.
- *Calibration and Maintenance:* This area is a maintenance area for calibration and small maintenance of the CGMP facility.
- *Utility Room:* This is the location for the CGMP utilities, i.e. R.O. water system, pure steam generator, WFI condenser, hot WFI storage tank, WFI distribution system for hot and ambient WFI, and a 1000 gal chilled water tank that is part of a 160 ton chiller system that distributes 40^{o}F throughout the facility at 270 gal/min.
- *Document Storage Area:* This area will be used to store all of the maintenance documents for the CGMP facility.

Figure 1. First floor layout of cGMP Facility


EQUIPMENT PLAN
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Buffer Prep Note:
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Purification Note:
General Note:
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Clean Technology Inc.
Project
Nebraska
BIOLOGICAL PROCESS DEVELOPMENT FACILITY CLEANROOM
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ARCHITECTURAL EQUIPMENT PLAN
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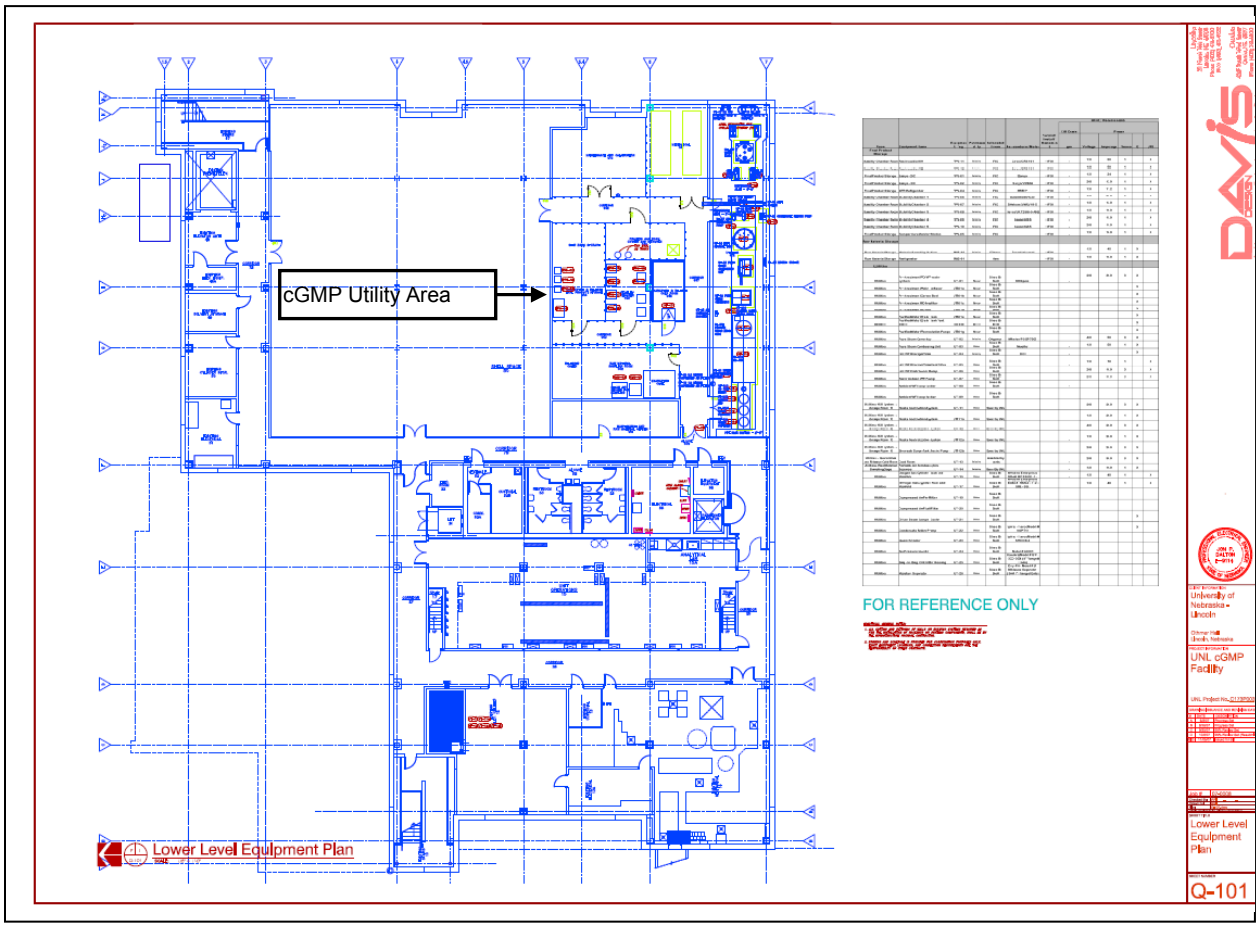
Figure 2. Basement Layout of cGMP



Photographs of the New cGMP Facility.

Figure 3. Class 10 Isolator System with an Isolet in the Aseptic Suite.

Figure 4. Fermentation Suite.

Figure 5. Buffer Prep Suite with 500 L Buffer Prep Tank.

Figure 6. Purification Suite.

Figure 7. Electrodionization (EDI) System for Pretreatement of Water Prior to the Pure Steam Generator.

Figure 8. Pure Steam Generator.

Figure 9. Water For Injection Distribution System and 1000 gal Water For Injection Storage Tank.

Figure 10. Water For Injection Distribution System.

Figure 11. Cleanroom Ductwork Directly Above the Aseptic Suite.

Figure 12. Air Handler No 1 in the Mezzanine above the Cleanrooms.

Project Funding For Construction

Funding Agency	Amount of Funding ($)
USAMRMC	$4,958,049
JVAP	$1,028,465
University of Nebraska-Lincoln	$5,179,226
Total Project Cost for Construction	$11,165,740

Key Accomplishment

There are *two* key accomplishments during this project. The first key accomplishment is publication of two manuscripts on the expression and purification and media optimization on the production of a monoclonal antibody against the serotype A botulinum neurotoxin. The second key accomplishment is the construction of a 13,000 sq ft cGMP facility with 6,000 sq ft of modular clean rooms. This cGMP facility will provide the capability to produce, Master and Working Cell Banks, production of Phase I and Phase II BDS (Bulk Drug Substance), and up to 1,500 manual fill of a FDP (Final Drug Product). As can be seen from the budget, the University of Nebraska-Lincoln provided a 46% funding match to construct this facility.

APPENDIX

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Protein Expression and Purification 37 (2004) 399–408
Protein Expression & Purification
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Production and purification of a chimeric monoclonal antibody against botulinum neurotoxin serotype A

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Abstract

Production of recombinant antibodies against botulinum neurotoxin is necessary for the development of a post-exposure treatment. CHO-DG44 cells were transfected with a plasmid encoding the light and heavy chains of a chimeric monoclonal antibody (S25) against botulism neurotoxin serotype A. Stable cell lines were obtained by dilution cloning and clones were shown to produce nearly equivalent levels of light and heavy chain antibody by an enzyme-linked immunosorbent assay (ELISA). In suspension culture, cells produced 35 μg/ml of chimeric antibody after 6 days, corresponding to a specific antibody productivity of 3.1 pg/cell/day. A method for the harvest and recovery of an antibody against botulism neurotoxin serotype A was investigated utilizing ethylenediamine-*N*,*N*′-tetra(methylphosphonic) acid (EDTPA) modified zirconia and MEP-hypercel, a hydrophobic charge interaction chromatography resin. Purification of the S25 antibody was compared to that achieved using rProtein A–Sepharose Fast Flow resin. After the direct load of culture supernatant, analysis by ELISA and gel electrophoresis showed that S25 antibody could be recovered at purities of 41 and 44%, from the EDTPA modified zirconia and MEP-hypercel columns, respectively. Although the purity obtained from each of these columns was low, the ability to withstand high column pressures and nearly 90% recovery of the antibody makes EDTPA modified zirconia well suited as an initial capture step. Combining the EDTPA modified zirconia and HCIC columns in series resulted in both purity and final product yield of 72%.

Keywords: Chinese hamster ovary; CHO; Mammalian; Cell culture; Zirconia; HCIC; Botulism; Botulism neurotoxin; BIAcore; Antibody

An increasing number of recombinant monoclonal antibodies are being developed for the treatment of medical conditions such as cancer, arthritis, and autoimmune diseases [1,2]. To meet the increased demand for monoclonal antibodies, all aspects of antibody production and purification need to be improved. Another potential use of monoclonal antibodies is the treatment for exposure to toxins, such as botulism neurotoxin (BoNT), one of the most poisonous substances known [3]. BoNT has been classified by the Centers of Disease Control (CDC) as one of the six highest risk threats for use in bioterrorism due to its potency, lethality, and ease of production [4]. BoNT is produced by the spore forming bacteria *Clostridium botulinum* and consists of seven serotypes (A–G) that cause the human disease botulism [5]. Botulism is characterized by flaccid paralysis and often results in death. The paralytic ability of the toxin has led to medical treatments for muscle conditions such as cervical distonias, cerebral palsy, and posttraumatic brain injury, in addition to its use for cosmetic purposes [6].

The potential use of BoNT in bioterrorism requires either a vaccine or other treatment for exposure.

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Currently, there are no small molecule drugs available to prevent botulism, although a pentavalent toxoid is available from the CDC. In addition to the pentavalent toxoid a recombinant vaccine is being developed [7,8]. Regardless of the availability of a recombinant vaccine, mass vaccination is unlikely due to the rarity of exposure and the fact the vaccination would prevent medical uses of BoNT. Antibodies, however, can be used for the post-exposure treatment of botulism. Equine antitoxin and human botulism immune globulin have been used for the post-exposure treatment of botulism [9,10]. Recombinant monoclonal antibodies are currently being developed for the treatment of botulism. Three monoclonal antibodies have been combined to neutralize 450,000 50% lethal doses of BoNT serotype A [3]. Half of the mice treated with a combination of three monoclonal antibodies were able to survive exposure to 450,000 times the amount of BoNT serotype A that would normally kill 50% of mice.

Post-exposure treatment of botulism would consist of a mixture of monoclonal antibodies against each of the seven BoNT serotypes. To produce large quantities of these recombinant monoclonal antibodies, it is necessary to improve production and purification methods. Several mammalian expression systems have been used for the high-level expression of monoclonal antibodies, including the use of dihydrofolate reductase (dhfr) deficient Chinese hamster ovary (CHO) cells [11]. These cells allow for amplification of gene expression upon the addition of methotrexate [12–15]. It has been shown that the productivity of CHO cells increases with gene copy number [16]. Upon selection in medium containing stepwise increases in methotrexate, CHO cells with monoclonal antibody productivities (qAb) as high as 100 pg/cell/day have been obtained [17].

After a production cell line is established, it is necessary to develop techniques for purification of the monoclonal antibody. The most common method of antibody purification is affinity chromatography based on Protein A or Protein G [18–23]. These purification methods are effective, but the sorbent is expensive and the leakage of Protein A results in the need for further purification processes. The expense and harsh elution conditions of affinity sorbents such Protein A have led to the search for alternative purification processes. These include hydrophobic interaction chromatography [24,25], hydroxyapatite [26], and ion-exchange chromatography [27]. Many of these purification techniques require significant treatment of the culture supernatant prior to purification. We have focused our efforts on purification using two different chromatography resins, MEP-hypercel, and ethylenediamine-*N*,*N*′-tetra(methylenephosphonic) acid (EDTPA) modified zirconia.

In the present study, we have developed and characterized production of a monoclonal antibody in a dhfr deficient CHO cell line and have analyzed a purification scheme that uses EDTPA modified zirconia as an initial capture and purification step followed by a secondary purification using MEP-hypercel, a hydrophobic charge interaction chromatography (HCIC) resin. EDTPA modified zirconia has previously been used for the separation of antibody from bovine serum albumin, a common component of mammalian cell culture medium [28,29]. Zirconia based resins provide excellent thermal and chemical stability compared to more typical resins. The zirconia surface is modified with EDTPA to block direct binding of antibody to the zirconia, which can lead to tailed elution bands and irreversible binding [30]. Hydrophobic charge induction chromatography (HCIC) has been used to purify antibodies directly from cell culture supernatant [31]. HCIC takes advantage of the pH behavior of the ionizable ligands. A decrease in the pH causes both the ligand and the protein to become positively charged, overcoming the hydrophobic interactions [32]. To obtain purified antibody against BoNT serotype A, CHO-DG44 cells were transfected with the genes for the light and heavy chains of the S25 antibody, and a purification scheme utilizing EDTPA modified zirconia and HCIC was compared to that obtained using a Protein A based resin.

Materials and methods

Cell line, media, transfection, and expression vectors

CHO-DG44 cells, which are dhfr negative, were obtained from Dr. Larry Chasin (Columbia University). This host cell line was maintained in α-MEM media (Invitrogen, Carlsbad, CA) supplemented with 8% fetal bovine serum (FBS) (Invitrogen). The pS25 plasmid (Fig. 1) was constructed by inserting the chimeric light and

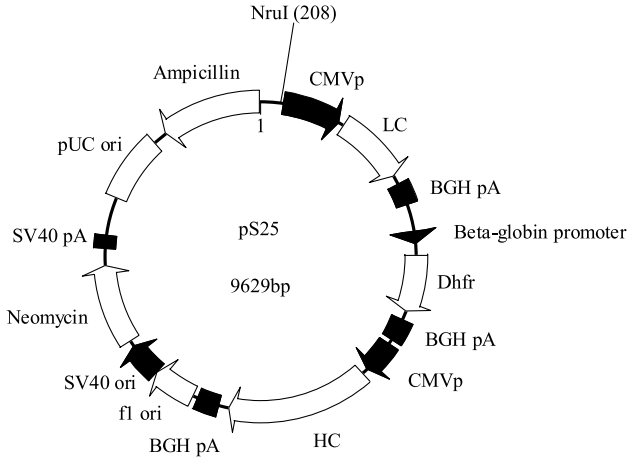

Fig. 1. Diagram of plasmid pS25. Plasmid contains the light (LC) and heavy chains (HC) of S25 antibody against BoNT serotype A, along with dhfr as a selectable marker.

heavy chain IgG genes against BoNT serotype A, along with the gene for dhfr into the plasmid pcDNA3.1(+) (Invitrogen).

CHO-DG44 cells were transfected with the pS25 plasmid using Lipofectamine 2000 (Invitrogen). Cells were seeded at 0.5 ml in 24-cell plates at a density of 2×10^5 cell/ml in α-MEM media containing 8% FBS and grown overnight. One microgram plasmid DNA and 0.5–2.0 μl Lipofectamine 2000 were combined in 0.1 ml Opti-MEM media (Invitrogen) and equilibrated for 20 min. Plasmid DNA was added to the transfection mix either uncut or linearized with *Nru*I placing the amplifiable gene (dhfr) between the heavy and light chains, increasing the likelihood that both the light and heavy chains would be amplified upon methotrexate addition. The DNA/Lipofectamine 2000 solution was added to the 24-well plates and the plates were incubated at 37 °C overnight. Stably transfected cells were selected in α-MEM media lacking ribonucleotides and deoxyribonucleotides, which prevent cells lacking dhfr from growing. Cells were passed several times and individual clones were obtained by dilution cloning at 0.5 cells/well in 96-well plates.

ELISA

The concentration of the whole antibody, as well as the concentration of the light and heavy chain portions, was determined using an enzyme-linked immunosorbent assay. Affinity purified rabbit anti-human IgG antibodies were diluted to 5 μg/ml in coating buffer (100 mM $NaHCO_3$, 100 mM NaCl, pH 9.3). One hundred microliters of diluted antibody was added to 96-well plates (Nunc, Rochester, NY) and incubated overnight at 4 °C. The plates were washed twice with Tris buffer (20 mM Tris–HCl, 50 mM NaCl, pH 7.2) containing 0.1% Tween 20 and then twice with Tris buffer alone. Blocking buffer (Tris buffer containing 0.5% BSA or casein) was added to the 96-well plates and incubated at 37 °C for 1 h. Supernatant samples were diluted in blocking buffer and samples were loaded into the 96-well plates in triplicate. Plates were incubated for 1 h at 37 °C and the washing procedure was repeated. One hundred microliters of a goat anti-human IgG-HRP conjugate antibody diluted to 0.5–2 μg/ml in the dilution buffer was added to the plates. The plates were incubated for 1 h at 37 °C and the washing procedure was repeated. Lastly, 100 μl of 1 mg/ml ABTS in ABTS buffer (Roche Applied Science, Indianapolis, IN) was added to the plates. The absorbance was determined at 405 nm using an ELx800 plate reader (Bio-Tek, Winooski, VT) after 30 min incubation. This procedure was used for whole antibody, heavy chain (Fc specific), and light chain (κ specific). Whole, Fc, and κ rabbit anti-human IgG coating antibodies and whole, Fc, and κ goat anti-human IgG-HRP conjugated antibodies were used in the ELISAs (Sigma, St. Louis, MO).

Transfer to suspension culture

After screening the clones for antibody production, nine clones that reached 0.5 μg/ml antibody after three days were transferred to suspension culture. Initially, cells were seeded in the spinner flasks at $2–3 \times 10^5$ viable cells/ml in CHO-S-SFMII media (Invitrogen) containing 1% FBS. The cells were then passed every 2–4 days into fresh media containing decreasing amount of FBS. After 8–10 passages, the cells were frozen in 1.5 ml aliquots in α-MEM media containing 10% FBS and 10% dimethyl sulfoxide (DMSO) at a cell density of 10^7 cells/ml.

Growth of cells in suspension culture

The CHO-DG44 S25 #56 cell line was grown in batch culture to analyze antibody production in suspension culture and to produce a sufficient amount of S25 antibody for purification and analysis. Frozen cells were resuspended in 40 ml CHO-S-SFMII at a seeding density of $3–4 \times 10^5$ cells/ml. The spinner flasks were incubated at 37 °C and 5% CO_2. The cells were fed every 3–4 days for several passages and were then seeded at 2×10^5 viable cell/ml in 350 ml CHO-S-SFMII media in a 1 L controlled spinner flask. The dissolved oxygen (DO), pH, and temperature were controlled using a Cellferm-Pro control system (DAS-GIP, Julich, Germany). The pH was controlled by addition of CO_2 and 1 M NaOH. Samples were taken every day and viability and cell density were determined by trypan blue exclusion and counting on a hemocytometer. Cell suspensions were centrifuged at 1200 rpm for 5 min and supernatant samples were frozen for later analysis.

Protein purification

EDTPA modified zirconia (Zirchrom, Anoka, MN), MEP-hypercel (Ciphergen, Fremont, CA), and rProtein A–Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ) resins were compared for the purification of S25 antibody. The supernatant from the CHO-DG44 S25 #56 cells was harvested after 6 days in batch culture. Supernatant was harvested by centrifugation at 300*g* for 5 min followed by a fivefold diafiltration with PBS (20 mM Na_2HPO_4, 150 mM NaCl, pH 7.4) using a Pellicon XL50 ultrafiltration device containing 0.005 m^2 of a 10 kDa MWCO Biomax membrane.

Supernatant samples were purified using an AKTA FPLC (Amersham Biosciences). Diafiltered samples were purified using a 100 mm × 4.6 mm diameter column containing Protein A–Sepharose Fast Flow resin (Amersham Biosciences). Alternatively, culture supernatant was directly loaded onto the rProtein A–Sepharose Fast Flow column, an MEP-hypercel column (100 mm × 4.6 mm diameter), or an EDTPA modified zirconia column (Zirchrom) (50 mm × 4.6 mm diameter). Prior to loading the

EDTPA modified zirconia column, the supernatant was diluted 1:1 in 40 mM Mes buffer containing 8 mM EDTPA (TCI America, Portland, OR).

After loading, the columns were washed with 5 column volumes (CV) of equilibration buffer. The rProtein A–Sepharose Fast Flow resin was loaded using PBS (pH 7.2) and eluted in 50 mM sodium citrate (pH 3.0). The MEP-hypercel column was equilibrated and washed with PBS (pH 7.2) and eluted using 50 mM sodium citrate (pH 4.0). The EDTPA modified zirconia column was equilibrated and washed with Mes buffer (20 mM Mes, 4 mM EDTPA, and 50 mM NaCl, pH 5.5) and eluted in Mes buffer containing 1 M NaCl. Samples were loaded and eluted at flowrates ranging from 0.25 to 0.5 ml/min. The pH of the elution was immediately increased to 7 using 500 mM Tris buffer (pH 9.0). The antibody was later concentrated and transferred into PBS by 10-fold diafiltration using a separate Pellicon XL50 ultrafiltration device. The S25 antibody was quickly frozen in liquid nitrogen at 1 mg/ml (BCA Assay) and was stored at −80 °C for long-term storage.

Bradford/BCA assays

The total protein content for the purified chimeric antibody and the culture supernatant were determined using either a Bradford reagent (Sigma) or BCA reagent (Pierce, Rockford, IL). The Bradford assay was used to determine the S25 antibody concentration in the culture supernatant and flowthrough, while the BCA assay was used to determine the concentration of the eluate. For the Bradford assay, a 1 ml sample was mixed with 1 ml Bradford Reagent (Sigma). The samples were incubated for 30 min at 37 °C and the absorbance at 595 nm was determined on a spectrophotometer. The BCA assay was used to determine the concentration of the S25 antibody product during the purification. For the BCA assay, 50 μl sample or standard was mixed with 1 ml BCA reagent mixture (Pierce), containing a 1:50 dilution of reagents A and B. The samples were incubated for 30 min at 37 °C and the absorbance at 562 nm was determined on a spectrophotometer. BSA, a major component of the growth media, was used as a standard for both assays. The BCA assay was used to determine the protein content of the eluate samples since IgG and BSA have a similar absorbance/mg in that assay. However, the Bradford assay was used to estimate protein content of other samples since components in the growth media interfered with the BCA assay.

SDS–PAGE/Western blotting

Samples were diluted in phosphate-buffered saline (PBS) to 60 and 20 μl loading buffer (0.5 M Tris–HCl, 20% SDS, 40% glycerol, 10% β-mercaptoethanol, and 0.1% bromophenol blue) was added. For non-reducing gels, the loading buffer lacked β-mercaptoethanol. Samples were boiled for 2 min and resolved on 10–12% Tris–glycine polyacrylamide gels (Invitrogen). The gels were run for 2–4 h at 125 V using an XCell SureLock Mini-Cell (Invitrogen) containing running buffer (50 mM Tris, 300 mM glycine, and 0.1% SDS). The gels were transferred to nitrocellulose in an XCell SureLock Mini-Cell module for 6 h at 25 V in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol, pH 8.3). Blots were blocked with 5% nonfat dried milk in TD buffer (140 mM NaCl, 5 mM KCl, 0.4 mM Na_2HPO_4, and 25 mM Tris) for 2 h at room temperature. Recombinant chimeric monoclonal antibody was detected by incubating with 0.5 μg/ml goat anti-human IgG (whole molecule specific) (Sigma) in 5% nonfat dried milk in TD buffer for 1 h at room temperature. The protein bands were detected by incubating with ECL (Amersham) and exposing to film. Human IgG (Sigma) was used as a positive control.

BIAcore activity assay

The S25 antibody affinity and binding kinetics were measured by surface plasmon resonance in a BIAcore (Biacore AB, Piscataway, NJ). The method for determination of antibody affinity was previously published [3]. Briefly, purified IgG in 10 mM acetate (pH 3.5–4.5) was coupled to a CM5 sensor chip using *N*-hydroxysuccinimide-*N*-ethyl-*N*′-(dimethylaminopropyl)-carbodiimide chemistry. The association constant (k_{on}) for purified BoNT serotype A Hc was measured under continuous flow of 15 μl/min. The dissociation constant (k_{off}) was determined at a high buffer flowrate of 30 μl/min to prevent rebinding. The equilibrium dissociation constant (K_d) was calculated as k_{off}/k_{on}.

Results and discussion

Isolation of recombinant CHO-DG44 cells with high S25 antibody production

Nearly 200 clones were screened for antibody using an ELISA specific for the Fc portion of the heavy chain of human IgG. Seventeen of the cell lines had antibody titers greater than 0.1 μg/ml, with one having an antibody titer greater than 2 μg/ml (Fig. 2). This clone (CHO-DG44 S25 #56) had the highest expression level throughout the selection process. Nine clones showed expression levels greater than 0.5 μg/ml after three days in adherent cultures (Fig. 3) using an Fc specific ELISA. Fig. 3 shows the concentrations of light, heavy, and whole antibody determined from separate ELISAs for each of the nine high expressing clones. It should be noted that only an Fc specific ELISA was performed for each of clones 160, 180, and 181. Similar light and heavy

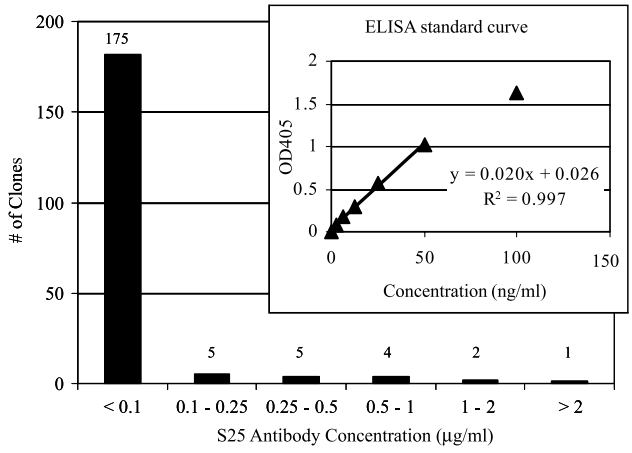

Fig. 2. Initial screening of CHO-DG44 transfectants using an Fc specific ELISA. One clone, CHO-DG44 S25 #56, had an S25 antibody content greater than 2 µg/ml.

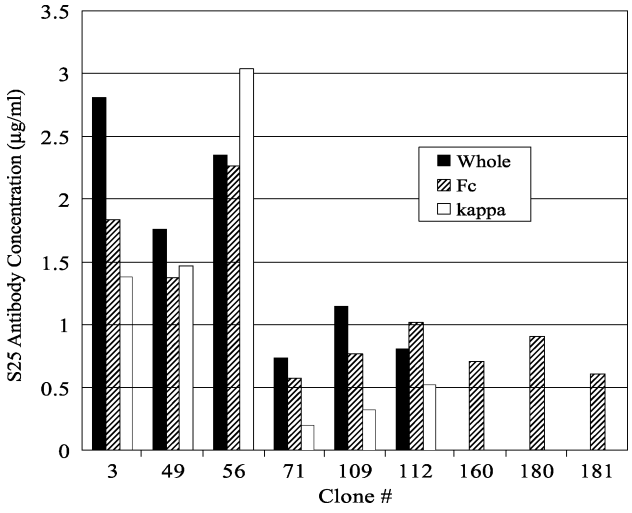

Fig. 3. Comparison of S25 antibody production in various CHO-DG44 S25 clones. Clones 3, 49, and 56 had the highest levels of expression. Clone #56 was chosen for initial production of S25 antibody. Whole IgG and κ concentrations were not determined for clones 160, 180, and 181. The concentration is the amount of S25 antibody based on either a whole molecule, Fc specific or κ specific ELISA.

chain antibody concentrations were determined for each of six clones tested. It is important to have similar expression levels of both the heavy and light chains to ensure full antibody is obtained upon purification. The cell line CHO-DG44 S25 #56 had light and heavy chain concentrations greater than 2 µg/ml and was therefore used for the initial production of S25 antibody in suspension culture. Each of these nine clones was transferred to increasing levels of methotrexate for gene amplification (data not shown) and into serum-free media (CHO-S-SFM II, Invitrogen) to ensure culture stability and productivity in suspension culture.

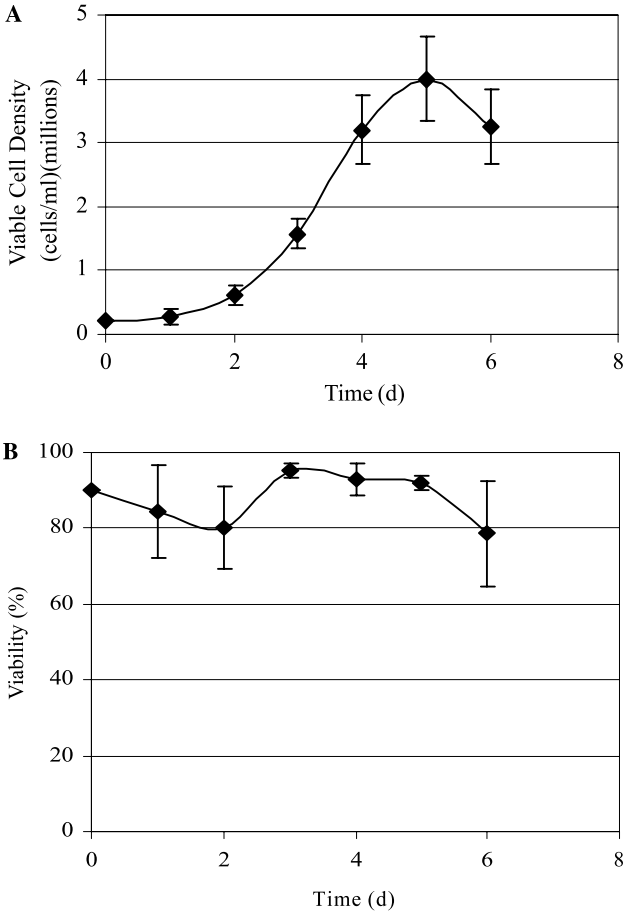

Fig. 4. Growth of CHO-DG44 S25 #56 cell line in suspension culture. (A) Viable cell density and (B) viability.

Production of a S25 antibody in batch culture

CHO-DG44 cells were transferred to suspension culture in CHO-S-SFM II and were grown in batch culture for the production of S25 anti-BoNT serotype A antibody. The CHO-DG44 S25 #56 cell line reached a maximum cell density of 4×10^6 cells/ml after 5 days in suspension culture (Fig. 4A). The viability of the cultures stayed above 90% until day 6 at which point it had dropped to 80% (Fig. 4B). This was confirmed by a corresponding decrease in the oxygen uptake rate. The cells reached a maximum growth rate (μ_{max}) of 0.95 day^{-1}, and the S25 antibody reached a final average concentration of 35 µg/ml, ranging from 21 to 53 µg/ml in four separate runs (Fig. 5). This corresponds to an average specific antibody productivity of 3.1 pg/cell/day, which is similar to that found for other recombinant antibodies prior to gene amplification [33].

Purification of S25 antibody using rProtein A–Sepharose fast flow resin

S25 antibody was purified from culture supernatant after a diafiltration step. Two hundred and ninety milliliters

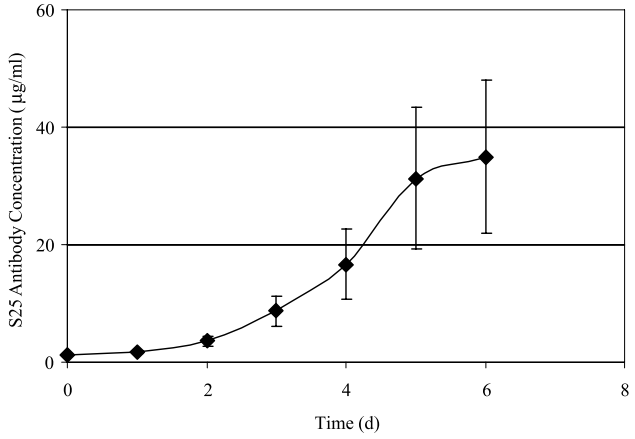

Fig. 5. S25 antibody production of CHO-DG44 S25 #56 cell line grown in CHO-S-SFM II media.

of supernatant was concentrated to 40 ml and was then transferred to PBS (pH 7.2) using a Pellicon XL50 ultrafiltration device. Diafiltered sample was loaded onto 2 ml rProtein A Fast Flow resin and eluted by gravity flow. The flowthrough was collected and the column was washed twice with 25 ml PBS (pH 7.2). The S25 antibody was eluted in 50 mM sodium citrate (pH 4), followed by a second elution at pH 3. Samples were analyzed by SDS–PAGE and Western blotting (Fig. 6). Faint bands can be observed for the light and heavy chains of the chimeric S25 antibody (lanes 6 and 7), corresponding to the supernatant from the CHO-DG44 S25 #56 cells and the dialyzed sample, respectively. Little antibody was lost in the flowthrough and wash step. Elution at reduced pH resulted in highly purified antibody. These results were confirmed by Western blotting using a goat anti-human IgG (whole molecule specific) (Sigma) (Fig. 6B).

The purification of S25 antibody with Protein A–Sepharose Fast Flow provided a 76% yield (Table 1). There was little loss of antibody during the ultrafiltration step. The S25 antibody had a purity of greater than 95%, which was confirmed by Coomassie stained SDS–PAGE (Fig. 6A). The total protein concentrations were determined by BCA assay for the eluate samples and a Bradford assay for the rest (Table 1).

BIAcore activity assay

S25 antibody activity was analyzed after purification using the rProtein A–Sepharose Fast Flow resin. The equilibrium binding kinetics were determined by BIAcore to ensure that the chimeric S25 antibody was active and had improved binding kinetics in comparison to the single chain variable fragment from which it was derived. The K_d of the S25 antibody was $2.0 \times 10^{-9}\,M^{-1}$, with a k_{on} of $6.0 \times 10^{5}\,M^{-1}s^{-1}$ and a k_{off} of $1.2 \times 10^{-3}\,s^{-1}$. This K_d is much better than that determined for the single chain variable fragment. The previously reported K_d of the single chain variable fragment was $7.30 \times 10^{-8}\,M^{-1}$, with a k_{on} of $1.10 \times 10^{4}\,M^{-1}s^{-1}$ and a k_{off} of $8.10 \times 10^{-4}\,s^{-1}$[3]. These values are also similar to those previously determined for both the S25 antibody and other antibodies [3,34,35].

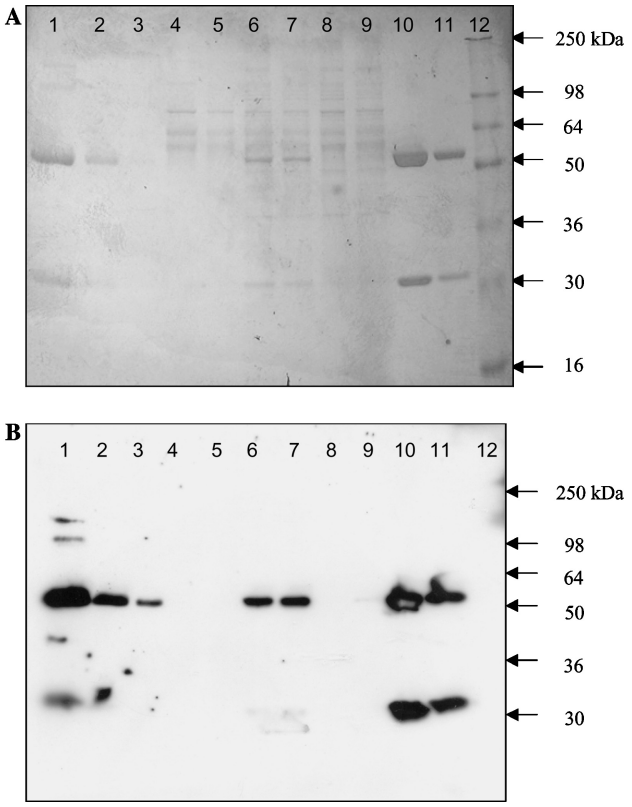

Fig. 6. Analysis of samples from S25 antibody purification using Protein A–Sepharose Fast Flow resin. (A) Coomassie stain and (B) Western blot (Anti-human IgG-HRP, whole molecule specific). (1) Human IgG (10 g), (2) Human IgG (2 µg), (3) Human IgG (0.4 µg), (4) CHO-S-SFM II media, (5) CHO-DG44 supernatant, (6) CHO-DG44 S25 supernatant, (7) Ultrafiltered using 10,000 kDa MWCO membrane, (8) Flowthrough, (9) Wash, (10) Eluate (pH 4.0), (11) Eluate (pH 3.0), and (12) See Blue Protein Standard.

Comparison of S25 antibody purified using EDTPA modified zirconia, MEP-hypercel, and rProtein A–Sepharose Fast Flow resins

Culture supernatant was directly loaded onto EDTPA modified zirconia, MEP-hypercel, and rProtein A–Sepharose Fast Flow chromatography columns (Figs. 7A–C). The loading of S25 antibody was well below the binding capacity of the rProtein A–Sepharose resin. The low maximum pressure drop (3 bar) of the rProtein A–Sepharose Fast Flow resin limits the flowrates to 90 cm/h. Approximately 6 mg of total protein containing 1.25 mg S25 antibody was loaded onto the column in 26.4 ml culture supernatant. The S25 antibody was eluted off the rProtein A column with

Table 1
Purification of S25 antibody using rProtein A–Sepharose Fast Flow resin

	Volume (ml)	S25 IgG (μg/ml)	Total S25 IgG (mg)	Total protein concentration (μg/ml)	Total protein (mg)	Yield (%)	Purity (%)
Supernatant	290	52.5	15.2	374	112.2	100	14
Diafiltration	39	373	14.5	2689	104.9	96	14
Flowthrough	39	3.6	0.14	1444	56.3	1	0
Wash 1	25	2.6	0.07	207	5.2	0	1
Wash 2	25	0	0.0	4	0.1	0	0
Elution 1 (pH 4)[a]	27.1	347	9.4	370	10.0	62	94
Elution 2 (pH 3)[a]	22.6	96.5	2.2	99	2.2	14	98
Eluate (Total)[a]	49.7	233	11.6	246	12.2	76	95

[a] BCA assay was used to determine the concentration of eluate samples. Absorbance/mg protein was nearly identical for IgG and BSA using the BCA assay, however components in the supernatant interfered with the BCA assay and therefore the Bradford assay was used to estimate protein concentration in the other samples.

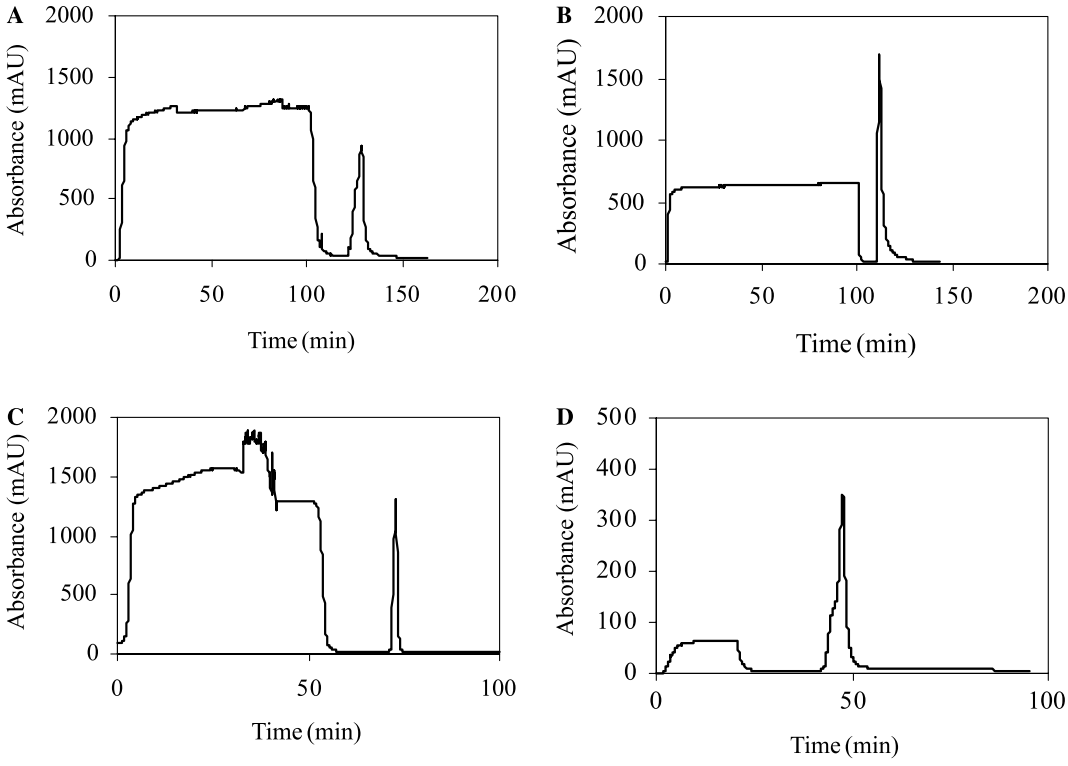

Fig. 7. Absorbance (280 nm) of S25 antibody separated on various chromatography columns. (A) rProtein A–Sepharose Fast Flow, (B) EDTPA modified zirconia, (C) MEP-hypercel, and (D) MEP-hypercel (loaded with elution from EDTPA modified zirconia column).

50 mM sodium citrate (pH 3.0) in 0.6 column volumes (CV) neutralized to pH 7.0 with 0.17 CV of 500 mM Tris base (Table 2). The Protein A–Sepharose Fast Flow column provided a yield of 75% as determined by a whole antibody ELISA. The purity of the antibody was determined to be 99% based on the concentration of IgG in the elution fraction determined by an ELISA divided by the total protein concentration determined by a BCA assay.

The EDTPA modified zirconia column was loaded with approximately 50 ml solution consisting of a 1:1 dilution of CHO-DG44 culture supernatant and Mes loading solution (40 mM Mes, 8 mM EDTPA) at a pH of 5.5. The EDTPA modified zirconia column has a smaller particle size (40 μm), which resulted in a higher initial pressure drop. However, the EDTPA modified zirconia resin can handle pressure drops exceeding 400 bar. The S25 antibody was eluted off the EDTPA modified zirconia column by increasing the NaCl concentration to 1 M in 20 mM Mes buffer containing 4 mM EDTPA. The S25 antibody eluted in 2.4 CV (Fig. 6B) and neutralized to pH 7.0 with 80 μl of 500 mM Tris base. The elution fraction had a S25 antibody concentration of 542 μg/ml and a protein concentration of

Table 2
Purification of S25 antibody using various chromatography resins

Sample	Volume (ml)	S25 IgG (μg/ml)	Total S25 IgG (mg)	Protein (μg/ml)	Total protein (mg)	Yield (%)	Purity (%)
rProtein A–Sepharose							
Supernatant	26.4	47.4	1.25	228	6.03	100	21
Flowthrough	26.4	0	0	81.4	2.15		
Wash	8.3	0	0	42.0	0.35		
Eluate[a]	1.28	731	0.94	738	0.95	75	99
rPEZ							
Supernatant	53.2	23.7	1.26	114	6.08		
Flowthrough	53.2	0	0	42.1	2.24		
Wash	8.3	0	0	20.0	0.17		
Eluate[a]	2.08	542	1.13	1312	2.73	89	41
MEP							
Supernatant	48.5	47.4	2.30	228	11.07		
Flowthrough	48.5	0	0	53.6	2.60		
Wash	8.3	0	0	25.3	0.21		
Eluate[a]	5.4	319	1.72	729	3.94	75	44
rPEZ/MEP							
Diafiltration	8.5	57.5	0.49	283	2.41		
Flowthrough	8.5	0	0	12.9	0.11		
Wash	8.3	0	0	7.8	0.06		
Eluate[a]	2.4	164	0.39	229	0.55	72	72

[a] BCA assay used to determine the concentrations of the eluate samples.

1312 μg/ml, which corresponds to a purity of 41%, a 1.9-fold increase. The yield for the EDTPA modified zirconia column was 89% (Table 2).

The MEP-hypercel column was loaded with approximately 50 ml supernatant from the CHO-DG44 S25 cells. The column was washed with 5 CV PBS (pH 7.2) and eluted using 50 mM sodium citrate (pH 4.0). The S25 antibody was eluted from the MEP-hypercel column in 2.7 CV (4.5 ml), similar to the EDTPA modified zirconia column. The elution fraction had an S25 antibody concentration of 319 μg/ml and a total protein concentration of 729 μg/ml, which corresponds to a purity of 44%, a 2.1-fold increase. The yield for the MEP-hypercel column was 75% which is similar to that achieved using the rProtein A column. The loss of S25 antibody appears to be due to irreversible binding onto the resin since there was little antibody in either the flowthrough or the wash fractions.

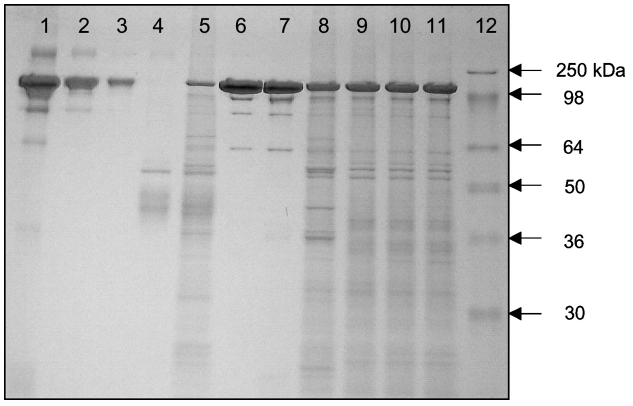

Fig. 8. Comparison of S25 antibody purification using rProtein A–Sepharose Fast Flow, MEP-hypercel, and EDTPA modified zirconia resins. (1) Human IgG (10 μg), (2) Human IgG (2 μg), (3) Human IgG (0.4 μg), (4) CHO-S-SFM II media, (5) CHO-DG44 S25 supernatant, (6) rProtein A pooled peak fraction (ultrafiltered load), (7) rProtein A pooled peak fraction, (8) EDTPA modified zirconia (9–11), MEP-hypercel fractions, and (12) See Blue Protein Standard.

Analysis of purification by Western blotting

A non-denaturing SDS–PAGE gel was run to compare purity of the EDTPA modified zirconia and MEP-hypercel purified samples to that purified using rProtein A–Sepharose Fast Flow (Fig. 8). Both rProtein A purified samples were very pure with no visible bands corresponding to non-IgG proteins. There are numerous bands that correspond to contaminating proteins from the CHO-DG44 S25 culture supernatant purified using EDTPA modified zirconia column. The S25 elution peak from the MEP-hypercel column is significantly broader than that obtained from the other columns and has a shoulder on the front. The peak was collected in three fractions, which were compared by SDS–PAGE (Fig. 8, lanes 9–11). All three fractions contain a high amount of contaminating proteins and therefore were combined. This shoulder suggests that an improvement in purity could be obtained by elution at several pH steps. Comparing the EDTPA modified zirconia and MEP-hypercel peaks it was observed that the contaminating bands in the MEP-hypercel column were different

from those occurring on the EDTPA modified zirconia column. As a result, the EDTPA modified zirconia column and MEP-hypercel column were run in series to improve the purity of S25 antibody.

Combination of EDTPA modified zirconia and MEP-hypercel purification

The EDTPA modified zirconia column was chosen as the first purification step since it can be operated at higher pressure drops and higher flowrates. In addition, the high antibody recovery makes it the preferred choice for an initial purification step. Neither the MEP-hypercel nor the EDTPA modified zirconia columns achieved purification efficiencies close to that achieved using the rProtein A–Sepharose Fast flow column. Numerous impurities that result in large bands in the S25 antibody elute taken from the EDTPA modified zirconia column were removed in the MEP-hypercel column. As a result, the product from the EDTPA modified zirconia column was loaded onto the MEP-hypercel column. The S25 antibody from the EDTPA modified zirconia column was dialyzed into PBS (pH 7.2) using an 8000 kDa MWCO dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA). The dialyzed sample was loaded onto the MEP-hypercel column followed by a 5 CV with PBS. The load onto the MEP-hypercel column was much less than the amount of supernatant previously loaded onto the column. The S25 antibody was eluted with 50 mM sodium citrate (pH 4.0).

The flowthrough of EDTPA modified zirconia purified S25 antibody sample loaded onto the MEP-hypercel column had an absorbance of about 70 mAU (Fig. 7D). This corresponds to protein that is being removed using the MEP-hypercel column. The S25 antibody is eluted at pH 4.0 and a peak height of 370 mAU is observed, which is much lower than those observed in the other columns due to the decreased antibody load. The antibody is eluted in 2 ml volume and 0.4 ml of 500 mM Tris base was immediately added to bring the pH to 7.0. The final S25 antibody concentration was 164 μg/ml and the final total protein concentration was 229 μg/ml, resulting in a final purification of 72%, a significant improvement to the purity obtained using the EDTPA modified zirconia column alone. The final yield for the EDTPA modified zirconia/MEP-hypercel purification was 72% which is just slightly less than that obtained from a single rProtein A column.

The S25 antibody purified using the EDTPA modified zirconia/MEP-hypercel columns in series was run on reducing SDS–PAGE gel, along with the samples purified using the EDTPA modified zirconia, rProtein A–Sepharose Fast Flow, and the MEP-hypercel alone (Fig. 9). Comparison of lanes 6, 7, and 9 shows the improvement in S25 antibody purity obtained after running both columns in series.

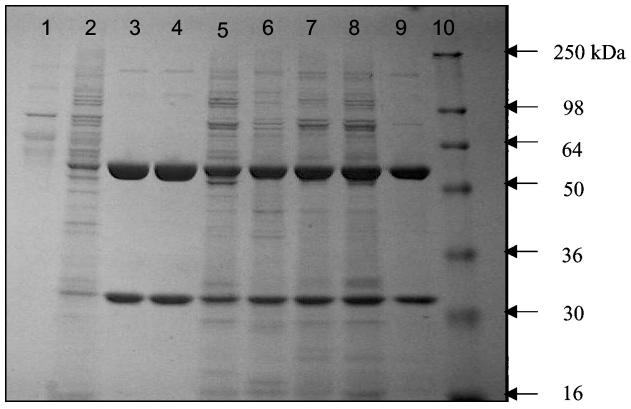

Fig. 9. Reducing gel of S25 antibody purified using various EDTPA modified zirconia and MEP-hypercel resins. (1) CHO-S-SFM II media, (2) CHO-DG44 S25 #56 supernatant, (3) rProtein A–Sepharose Fast Flow resin (dialyzed load), (4) rProtein A–Sepharose Fast Flow, (5) EDTPA modified zirconia, (6) MEP-hypercel, (7) EDTPA modified zirconia #2, (8) Dialyzed sample from EDTPA modified zirconia #2, (9) EDTPA modified zirconia/MEP-hypercel, and (10) See Blue Protein Standard.

Conclusion

An antibody against BoNT serotype A was produced in CHO-DG44 cells and was then purified. The combination of EDTPA modified zirconia and MEP-hypercel provided an initial purification of monoclonal antibodies, but further downstream processing steps or improvements in separation conditions are needed to approach the purity achieved using a single Protein A resin. While EDTPA modified zirconia does not approach Protein A resins for purity, the ability to operate at increased pressures, the high yield, and the ease of cleaning make it an ideal capture step for the purification of monoclonal antibodies from culture supernatant. In addition, EDTPA modified zirconia and MEP-hypercel prove to be complementary purification steps as demonstrated by the large increase in purity obtained when running these steps in series.

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ORIGINAL RESEARCH

Developement of serum-free media in CHO-DG44 cells using a central composite statistical design

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Abstract A serum free medium was developed for the production of recombinant antibody against Botulinum A (BoNTA) using dihydrofolate reductase deficient Chinese Hamster Ovary Cells (CHO-DG44) in suspension culture. An initial control basal medium was prepared, which was similar in composition to HAM's F12: IMDM (1:1) supplemented with insulin, transeferrin, selenium and a lipid mixture. The vitamin concentration of the basal medium was twice that of HAM's F12: IMDM (1:1). CHO-DG44 cells expressing S25 antibody grew from 2×10^5 cells to maximum cell density of 1.04×10^6 cells/ml after 5 days in this control medium. A central composite design was used to identify optimal levels and interaction among five groups of medium components. These five groups were glutamine, Essential Amino Acids (EAA), Non Essential Amino Acids (NEAA), Insulin, Transferrin, Selenium (ITS), and lipids. Fifty experiments were carried out in four batches, with two controls in each batch. There was little effect of ITS and Lipid concentrations over the range studied, and glutamine concentration showed a strong interaction with EAA. The optimal concentrations of the variables studied were 2.5 mM Glutamine, 7.4 mM (2×) EAA, 1.4 mM (0.5×) NEAA, 1× ITS supplement, 0.7× Lipids supplement. The maximum viable cell density attained in the optimized medium was 1.4×10^6 cells/ml, a 35% improvement over the control culture, while the final antibody titer attained was 22 ± 3.4 μg/mL, a 50% improvement.

Keywords Response surface method · Media optimization

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Introduction

Botulinum neurotoxin, produced by the anaerobic *Clostridium botulinum*, is one of the most potent toxins known to humans (Franz et al. 1997; Gill 1982). *Clostridium botulinum* produces seven antigenically distinct neurotoxins (A–G) differentiated serologically by specific neutralization (Hatheway 1990). The active neurotoxin is synthesized as two polypeptide chains, a heavy chain (100 KDa) and a light chain (50 KDa), connected via disulfide linkage (DasGupta and Sathyamoorthy 1984; Syuto and

Kubo 1981). Recombinant monoclonal antibodies (mAb) can neutralize the effects of BoNT A without requiring human donors for plasmapheresis (Lang et al. 1993). Potent neutralizing monoclonal antibodies were identified recently, characterized, cloned and expressed in Chinese Hamster Ovary (CHO) cells to yield humanized mAbs (Nowakowski et al. 2002).

The large-scale, commercial production of therapeutically important proteins from rCHO cells typically involves a suspension culture-based manufacturing process (Sinacore et al. 1996). It is desirable to use serum-free medium in suspension culture because serum can cause problems in subsequent processes (Glassy et al. 1988; Keen and Rapson 1995). However, there is no universal serum-free medium applicable to all cell lines. There is need to develop specific medium suitable for each cell line (Hayter et al. 1991; Zang et al. 1995).

The medium used for animal cell culture is very complex. Further, as the impact of medium components on cell growth or product synthesis is rather difficult to fully elucidate, statistical methods are adopted to develop a medium for cell culture that offers optimal viable cell density and product formation. The traditional one-factor-at-a-time approach to optimization is time-consuming and incapable of reaching the true optimum especially because of interactions among the factors that influence the growth process. Moreover, this approach assumes that the various growth parameters do not interact and the process response is a direct function of a single varied parameter (Castro et al. 1992; Freshney 1994). In reality, the observed behavior of growth results from the interactive influences of the various variables (Chen et al. 2002). To be effective, optimization requires statistical methods that take these interactions into account.

Response surface methodology (RSM), an experimental strategy for seeking the optimum conditions for a multivariable system, is a much more efficient technique for optimization (Box et al. 1978). RSM comprises of mathematical and statistical procedures that can be used to study relationships between one or more responses and a number of independent variables. In addition to analyzing the effects of independent variables, this experimental methodology generates a mathematical model that accurately describes the overall process (Myers and Montgomery 1995; Senanayake and Shahidi 2002). RSM has been employed to solve multivariate problems and optimize responses in many types of experiments (Maddox and Richert 1977; Giovanni 1983; Houng et al. 1989). In this approach, concentrations of medium components are the variables; each variable refers to some base value and varies in a certain pattern. This pattern is designed by using statistical methods to yield the most information by a minimum number of experiments.

In this study, we have adopted a RSM approach to locate the optimum levels of Glutamine, Essential Amino Acids (EAA), Non-Essential Amino Acids (NEAA), Insulin, Transferrin, Selenium (ITS), and Lipids. Our objective is to gain insight into the interactions among these factors that significantly impact the response and viable cell density. Glutamine acts as the primary source of nitrogen as well as an additional carbon and energy source. It contributes precursors to the formation of the major intracellular binding blocks: amino acids, proteins. Approximately 30–65% of the cell energy requirement is derived from glutamine metabolism (Zielke et al. 1984; Reitzer et al. 1979). Amino acids are primary sources of nitrogen that protect cells from nutrient deprivation (Franek and Sramkova 1996), elevated osmolarity (Øyaas et al. 1994) and elevated pCO_2 (de Zengotitia et al. 2002). Insulin serves as a growth and maintenance factor and is considered to be important for serum-free cultures (Schubert 1979). Insulin stimulates uridine and glucose uptake and synthesis of RNA, proteins and lipids; it also increases fatty acid and glycogen synthesis (Mather and Sato 1979). Transferrin is one of the most essential growth-promoting supplements in serum-free medium, and its omission causes severe inhibition of cell growth (Kovar and Franek 1984). Transferrin is an iron binding glycoprotein that interacts with surface receptors. It is closely related to the transport of iron across the plasma membrane (Bretscher 1985). Transferrin has additional in-vitro functions, e.g., chelation of deleterious trace materials, that are unlikely replaced by other components. Selenium is a trace element essential for mammalian cell cultures (Nielsen et al. 1981); its mechanism is poorly understood. There is evidence that selenium enhances growth rate in serum free-cultures (Darfler and Insel 1983). Lipids are required for proliferation, differentiation, and antibody secretion. They play a major role in the cell membrane which is composed of a

phospholipid bilayer, and help in the transmission of nutrients into the cell and excretion of proteins out the cell (Farrant et al. 1984). The major functions exhibited by the variables motivated us to choose the response surface methodology in order to observe the higher order interactions that would maximize the response. Results were analyzed statistically by SAS and optimum conditions were selected graphically. Interactions among these factors were also examined.

Materials and methods

Cell line and medium

CHO-DG44 cells, which are dhfr negative, were obtained as a gift from Dr. Larry Chasin (Columbia University). The parental cell line was obtained by weaning CHO-DG44 from the serum according to standard cell culture techniques. The base medium used during the weaning process was a commercial serum-free medium, CHO-S-SFMII, known to contain animal-derived proteins and hydrolysates. The process of weaning CHO-DG44 of its serum dependence lasted approximately 4–5 months. The resulting cell line was used as the starting point for all subsequent development efforts including recombinant cell line generation, medium development studies. The recombinant cell line was constructed by inserting the chimeric light and heavy chain IgG genes against BoNT serotype A, along with the gene for dhfr into the plasmid pcDNA3.1(+) (Invitrogen, Carlsbad/CA) and the procedures are detailed elsewhere (Mowry et al. 2004). Recombinant cell lines were derived from our parent cell line using standard molecular biology techniques.

Medium

The basal medium was prepared similar to the HAM's F12: IMDM (1:1) medium excluding Hypoxanthine and Thymidine, by adding components separately. The concentration of the inorganic salts and other components such as linoleic acid, lipoic acid, phenol red, putrescine 2HCl, sodium pyruvate, and HEPES is the same as with HAM's F12:IMDM (1:1). The concentrations of the glucose and glutamine in the starting basal medium were 4 g/L and 4 mM, respectively. For amino acids the medium was supplemented with 1.75× of EAA and 1.75× of NEAA, which come from Gibco as 50× and 100× solutions respectively. The concentrations of individual amino acids in EAA (50×) and NEAA (100×) solutions are given in Table 1. The additional components added to the basal medium are vitamins, the concentrations of which were double those in HAM's F12:IMDM (1:1), 1× (ITS), and 0.7× Lipids Supplement, which come as 100× solutions from Gibco. The concentration of the individual components in ITS and Lipids Supplements are given in the Table 1. The composition of the complete basal medium is given in Table 2.

Table 1 Composition of the individual components in mg/L in the solutions EAA, NEAA, ITS, Lipids, concentrated solutions from Gibco

(50×) EAA [mg/L]	(100×) NEAA [mg/L]	(100×) ITS [mg/L]	(100×) Lipids [mg/L]
L-Arginine [6320]	L-Alanine [890]	Insulin [1000]	Arachidonic acid [2]
L-Cystine[1200]	L-Asparagine[1320]	Transferrin [0.67]	Cholestrol [220]
L-Histidine × HCl × H_2O [2100]	L-Aspartic acid [1330]	Selenium [0.55]	DL-alpha-tocopherol-acetate [70]
L-Isoleucine [2620]	L-Glutamic acid [1470]		Linoleic acid [10]
L-Leucine [2620]	Glycine [750]		Linolenic acid [10]
L-Lysine HCl [3625]	L-Proline [1150]		Myristic acid [10]
L-Methionine [755]	L-serine [1050]		Oleic acid [10]
L-Phenylalanine [1650]			Palitoelic acid [10]
L-Threonine [2380]			Palmitic acid [10]
L-Tryptophan [510]			Pluronic [0.1%]
L-Tyrosine [1800]			Stearic acid [10]
L-Valine [2340]			Tween 80 [2200]

Table 2 Concentrations of the components in the control medium

Components	Composition [mg/mL]	Components (contd.)	Composition [mg/mL]
$CaCl_2$ (anhyd.)	99.1	L-Phenylalanine	57.7
$CuSO_4 \times 5H_2O$	0.00125	L-Proline	20.12
$FeSO_4 \times 7H_2O$	0.415	L-Serine	18.37
KCl	276.8	L-Threonine	83.3
$MgCl_2$ (anhyd.)	28.61	L-Tryptophan	17.8
NaCl	5500	L-Tyrosine $\times$ 2Na $\times 2H_2O$	63
$NaHCO_3$	2100	L-Valine	81.9
Na_2HPO_4 (anhyd.)	71	Biotin	0.02
$Na_2HPO_4 \times$ H2O	62.5	D-Ca Pantothenate	4
$ZnSO_4 \times 7H_2O$	0.43	Choline chloride	18
KNO_3	0.038	Folic acid	4
$MgSO_4$ (anhyd.)	50.8	i-Inositol	25.2
Na_2SeO_3	0.0085	Niacinamide	4
D-glucose	4000	Pyridoxine HCl	4
Linoleic acid	0.04	Riboflavin	0.4
Lipoic acid	0.105	Thiamine HCl	4
Phenol Red	8.1	Vitamin B12	1.413
Putrescine 2HCl	0.0805	Insulin	10
Sodium pyruvate	110	Transferrin	5.5
HEPES	2979	Sodium selenite	0.0134
L-Alanine	15.57	Arachidonic acid	0.014
L-Arginine $\times$ HCl	221.2	Cholesterol	1.54
L-Asparagine $\times H_2O$	23.1	DL-alpha-tocopherol-acetate	0.49
L-Aspartic acid	23.27	Linoleic acid	0.07
L-Cystine $\times$ 2HCl	42	Linolenic acid	0.07
L-Glutamic acid	25.72	Myristic acid	0.07
L-Glutamine	584	Oleic acid	0.07
Glycine	13.12	Palitoelic acid	0.07
L-Histidine $\times$ HCl $\times H_2O$	73.5	Palmitic acid	0.07
L-Isoleucine	91.7	Stearic acid	0.07
L-Leucine	91.7	Tween 80	15.4
L-Lysine $\times$ HCl	126.88	Pluronic	0.10%

Cell culture

The cell cultivation was performed in 37°C humidified incubators supplemented with 5% carbon dioxide. The seeding density was 2×10^5 cells/mL, and cell counts were performed every 4 days. The number of cells were determined using a hemocytometer. Spheroids would be enzymatically dissociated when spherical aggregates were observed. Approximately, 1.7 mL of sample were harvested from spinners and placed in a 2.0 mL microtube and centrifuged at 1,200 rpm for 6 min; 1.5 mL supernatant was saved for antibody assays. Hundred microliters (μL) of trypsin solution (2.5% (w/v) Trypsin in PBS) was added to resuspend the cells of 400 μl. The cells were incubated at room temperature for 15 min and the cell density and viability were then determined by the trypan blue exclusion method.

Antibody assay

The concentration of the whole antibody, as well as the concentration of the light and heavy chain portions, was determined by an enzyme-linked

immunosorbent assay. Affinity purified rabbit anti-human IgG antibodies were diluted to 5 μg/mL in a coating buffer consisting of 100 mM $NaHCO_3$ and 100 mM NaCl (pH = 9.3). About 100 μL diluted antibody was added to 96 well plates (Nunc) and incubated overnight at 4°C. The plates were washed twice in a Tris buffer (20 mM Tris–HCl, 50 mM NaCl, pH = 7.2) containing 0.1% Tween 20, and washed twice in the Tris buffer alone. Blocking buffer (Tris buffer with 0.5% BSA) was added to the 96 well plates and incubated at 37°C for 1 h. Supernatant samples were diluted in the blocking buffer and samples were loaded into the 96 well plates in triplicate. Plates were incubated for 1 h at 37°C and the washing procedure was repeated. Hundred microliters (μL) of a goat anti-human IgG-HRP conjugate antibody diluted to 0.5–2 mg/mL in the dilution buffer was added to the plates. The plates were incubated for 1 h at 37°C and the washing procedure was repeated. Finally, 100 mL of 50 μg/mL ABTS in ABTS buffer (Roche) was added to the plates. The absorbance was determined at 405 nm using an ELx800 plate reader (Bio-Tek). This procedure was used for the whole antibody, the heavy chain (Fc specific), and the light chain (κ specific). In sandwich ELISA assays, rabbit anti-human IgG antibodies raised against the whole molecule, Fab-specific and kappa-chain specific, respectively, were used to coat the ELISA plates. HRP-conjugated rabbit anti-human IgG antibodies raised against the whole molecule, Fab-specific and kappa-chain specific, respectively, were used in the detection step of the ELISA assays.

Experimental design

Response surface methodology (RSM) was used to determine the influence of some medium components on the response of viable cell density. Our assumption is that since our product is growth-associated, increase in viable cell density will ultimately increase the antibody production. Theoretical and fundamental aspects of RSM have been extensively discussed elsewhere (Myers and Montgomery 1995). The experimental design adopted Box's central composite design for five variables at five levels each. The five independent variables were $X1$ = Glutamine, $X2$ = EAA, $X3$ = NEAA, $X4$ = ITS, $X5$ = Lipids. The independent variable coded regions were $-\alpha$ (-2, Lowest Level), -1, 0 (middle level), 1, and $+\alpha$ (2, highest level). The actual values, which were chosen from preliminary studies, and the corresponding coded and uncoded values of the five independent variables are given in Table 3. The complete design has 42 experimental points, which including eight replications of the center point. The treatment combinations and observed responses are presented in Table 4. The 50 experimental medium runs were prepared in random order and the experiments were performed in four batches. The dependent variable (Y) was viable cell density and was assumed to be affected by the five independent variables. Based on data from this design, we fit a second order or higher order polynomial regression model described as follows:

Table 3 Actual factor levels corresponding to coded factor levels

Level	Symbol	Actual factor level at coded factor level of				
		−2	−1	0	1	2
Glutamine	$X1$	1 mM	2.5 mM	4 mM	5.5 mM	7 mM
EAA	$X2$	0×	0.5×	1×	1.5×	2×
NEAA	$X3$	0.25×	1×	1.75×	2.5×	3.25×
ITS	$X4$	0.25×	1×	1.75×	2.5×	3.25×
LIPIDS	$X5$	0.1×	0.4×	0.7×	1×	1.3×

$$Y = b_l + \sum_{i=1}^{k} biXi + \sum_{i=1}^{k} b_{ii}X_i^2 + \sum_{i}\sum_{j} bijXiXj + \epsilon$$

where

Y = Viable cell density
b_l = Intercept for block l
X = Factors ($X1$ = Glutamine, $X2$ = EAA, $X3$ = NEAA, $X4$ = ITS, $X5$ = Lipids)
b_z = Regression coefficient (z = i, ii or ij, *where* $i < j$)
ε = Residual error
k = 1,2,3…

Statistical analysis

Using ordinary least squares the regression model was fit to evaluate, the explanatory variables regarding linear, interaction, and quadratic effects of coded

Table 4 Treatment combinations with variables in coded values and the values of response

Run	*X*1	*X*2	*X*3	*X*4	*X*5	*Y*
1	1	1	−1	−1	1	8.1E + 05
2	1	1	−1	1	−1	7.2E + 05
3	−1	−1	−1	−1	−1	6.5E + 05
4	0	0	0	2	0	8.3E + 05
5	−1	1	1	−1	−1	1.0E + 06
6	1	−1	1	−1	1	8.0E + 05
7	2	0	0	0	0	7.3E + 05
8	0	0	0	0	2	7.1E + 05
9	1	1	−1	1	1	5.4E + 05
10	0	0	0	0	0	9.5E + 05
11	1	−1	−1	1	1	6.6E + 05
12	0	0	0	0	0	9.3E + 05
13	−1	−1	1	1	1	8.40E + 05
14	1	−1	1	1	−1	8.80E + 05
15	1	−1	1	1	1	6.25E + 05
16	−1	−1	1	−1	−1	7.00E + 05
17	0	0	0	0	0	1.18E + 06
18	−1	−1	−1	1	1	8.15E + 05
19	0	0	0	0	−2	1.15E + 06
20	1	−1	−1	1	−1	6.55E + 05
21	0	0	0	0	0	1.16E + 06
22	−1	−1	−1	−1	1	2.00E + 05
23	−2	0	0	0	0	1.52E + 06
24	0	0	0	−2	0	5.15E + 05
25	0	−2	0	0	0	3.10E + 05
26	−1	−1	1	1	−1	8.20E + 05
27	1	−1	−1	−1	1	5.10E + 05
28	1	1	1	−1	−1	8.10E + 05
29	−1	1	−1	−1	−1	7.85E + 05
30	0	0	0	0	0	1.18E + 06
31	−1	−1	−1	1	−1	8.30E + 05
32	0	0	2	0	0	9.55E + 05
33	−1	1	1	−1	1	1.29E + 06
34	0	0	−2	0	0	1.80E + 05
35	1	1	−1	−1	−1	2.00E + 05
36	1	1	1	−1	1	2.20E + 05
37	0	0	0	0	0	1.21E + 06
38	−1	1	1	1	1	1.03E + 06
39	−1	1	−1	1	−1	9.00E + 05
40	1	−1	1	−1	−1	9.85E + 05
41	−1	1	1	1	−1	8.65E + 05
42	1	−1	−1	−1	−1	8.75E + 05
43	1	1	1	1	−1	7.70E + 05
44	1	1	1	1	1	1.08E + 06
45	0	0	0	0	0	9.90E + 05
46	−1	1	−1	−1	1	9.35E + 05
47	−1	1	−1	1	1	8.55E + 05
48	−1	−1	1	−1	1	1.00E + 06
49	0	2	0	0	0	8.90E + 05
50	0	0	0	0	0	9.85E + 05

Where α = '2', Response (Y) = Viable cell density in cells/mL, '−1', '0', '+1' are coded factorial levels

levels of Glutamine, EAA, NEAA, ITS, Lipids on cell density. The R^2 value was used to evaluate model sufficiency and the α-level was set as 5%, at which point every term in the selected model should be significant. The reduced model was evaluated using the R^2. Lack of fit was used to attempt to find optimal conditions for all the variables maximizing the cell density. Canonical analysis was then used to evaluate the nature of the stationary point (maximum, minimum or saddle) and to find the ridge of steepest ascent. Further experiments were carried out in the direction of the maximum response along with alternate experiments where Glutamine was set to different coded levels from '0' to '−3', keeping EAA constant at coded level '2' and '4'. All statistical computations were done using SAS/STAT procedures, and optimum conditions were found through SAS data-step programming. Response surface plots were generated by SAS/GRAPH.

Results and discussion

Regression analysis revealed that linear (X_i) and quadratic effects ($X_i \times Xi$) were more significant than cross product interactions ($X_i \times X_j$), as based on the *p*-values obtained (Table 5). Among all independent variables, Glutamine (negative effect, X_1: −2.79) and NEAA (positive effect, X_3: 3.07) had the greatest effects on the cell density, while EAA showed an effect when combined with Glutamine ($X_2 \times X_1$). Among the pairwise interactions, EAA and Glutamine exhibited the greatest effect. Although NEAA squared ($X_3 \times X_3$) and NEAA (X_3) by itself were significant, they did not have a great effect when

Table 5 The *t*- and *p*- values of full model with *X*1, *X*2, *X*3, *X*4, *X*5 as independent variables

Parameter	*t*-value	*p*-value
Intercept	13.75	<0.001
*X*1	−2.79	0.0092
*X*2	1.53	0.1376
*X*3	3.07	0.0046
*X*4	1.2	0.2404
*X*5	−0.81	0.425
*X*1 × *X*1	0.66	0.5167
*X*2 × *X*1	−2.1	0.1446
*X*2 × *X*2	−2.67	0.0124
*X*3 × *X*1	−0.27	0.7855
*X*3 × *X*2	−0.1	0.9206
*X*3 × *X*3	−2.86	0.0078
*X*4 × *X*1	0.31	0.758
*X*4 × *X*2	0.22	0.8304
*X*4 × *X*3	−0.74	0.4662
*X*4 × *X*4	−2.21	0.0352
*X*5 × *X*1	−0.8	0.4292
*X*5 × *X*2	1.3	0.2026
*X*5 × *X*3	0.25	0.8061
*X*5 × *X*4	0.2	0.8427
*X*5 × *X*5	−0.58	0.5675
Linear		0.0046
Quadratic		0.0055
Cross Product		0.6621

R^2 = 0.6339 for the total model

compared with the other variables, as judged by the *p*-value. The response surface plots were then plotted to see the effect of EAA and NEAA (Fig. 1), EAA and Glutamine (Fig. 2), NEAA and Glutamine (Fig. 3) on the response which is the viable cell density (*Y*). ITS and Lipids were found to have no effect. The R^2 value for the total model is 0.6339. To simplify the model, the variables of ITS and Lipids were removed from the model and the data were re-analyzed using the reduced model. The polynomial regression model used for three variables was

$$Y = b_o + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_{11}^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + \epsilon$$

When the variables ITS and Lipids were kept constant, the lack of fit is found to be insignificant, suggesting that the model was adequate to explain the effect of these three variables on the response. The R^2 value of the reduced model decreases to 0.502, as denoted in Table 6. As Table 6 shows, the Glutamine (*X*1) had significant linear effects and interacted with EAA (*X*2). EAA and NEAA had significant quadratic effects (*X*2 × *X*2 and *X*3 × *X*3) while NEAA (*X*3) also had a linear effect on the cell density.

Canonical analysis

Canonical analysis is a mathematical approach used to examine the overall shape of the response surface and to determine if the estimated response point is a maximum, minimum or a saddle point. If the stationary point is maximum or minimum, a corresponding increase or decrease will result in the response. In the case of a saddle point, the response may increase or decrease when we move away from

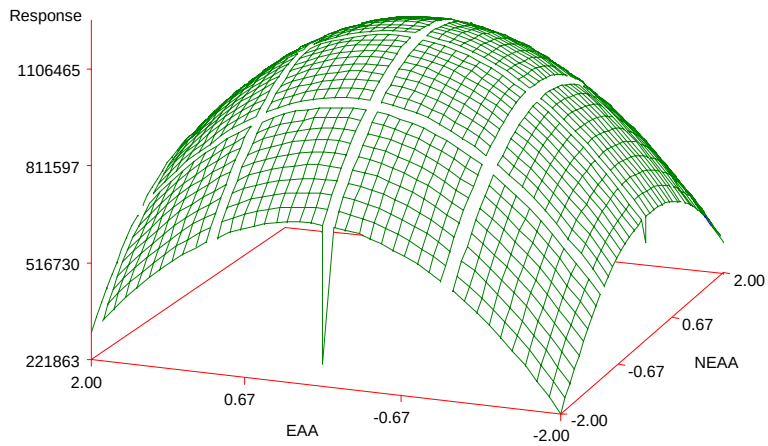

Fig. 1 Response surface plot showing the effect of EAA, NEAA, and their mutual effect on the response (viable cell density). Other variables were held at zero level

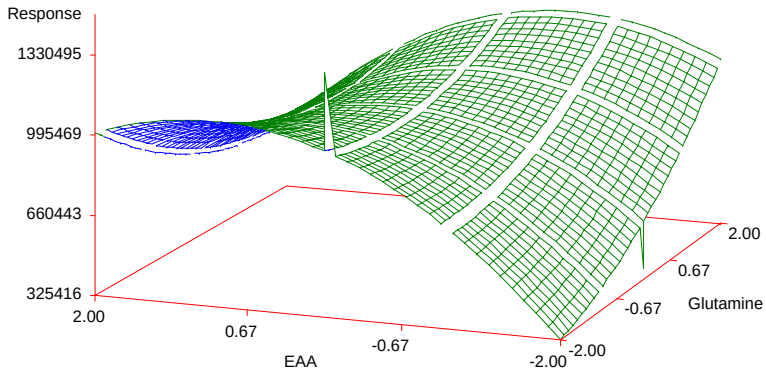

Fig. 2 Response surface plot showing the effect of EAA, glutamine and their mutual effect on the response (viable cell density). Other variables were held at zero level

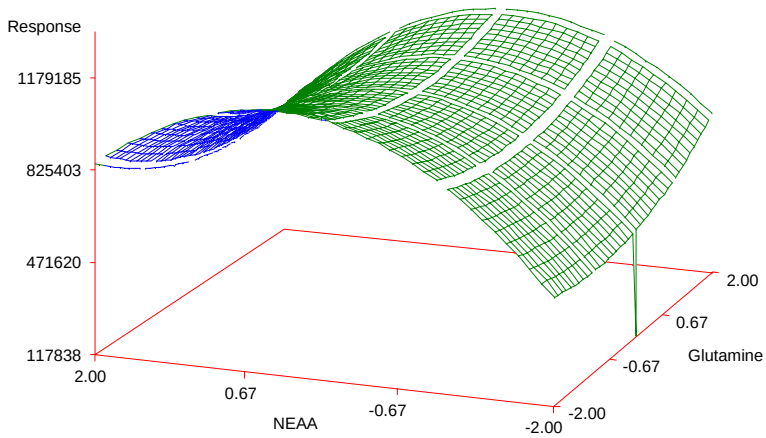

Fig. 3 Response surface plot showing the effect of NEAA, glutamine and their mutual effect on the response (viable cell density). Other variables were held at zero level

Table 6 The *t*- and *p*- values of the reduced model with *X*1, *X*2, *X*3 as independent variables

Parameter	*t*-value	*p*-value
Intercept	15.56	<0.0001
*X*1	−2.81	0.0076
*X*2	1.54	0.132
*X*3	3.1	0.0036
*X*1 × *X*1	0.66	0.5123
*X*2 × *X*1	−2.11	0.0407
*X*2 × *X*2	−2.69	0.0105
*X*3 × *X*1	−0.28	0.7835
*X*3 × *X*2	−0.1	0.9199
*X*3 × *X*3	−2.88	0.0064
Linear		0.001
Quadratic		0.0035
Cross product		0.2242

R^2 = 0.5022 for the reduced model

the stationary point, depending on which direction is taken. Maximizing the viable cell density is of interest; however the stationary point was a saddle point, so we move on the ridge in the direction to get the maximum response.

The points on the ridge that increased the response were found using the RIDGEMAX option of the SAS/RSREG procedure, and are shown in Table 7. From Tables 6 and 7, glutamine showed a negative effect on cell density while EAA, NEAA showed a positive effect. Therefore the glutamine values for the ridge moved in the negative direction and the values for EAA and NEAA moved in the positive direction. Following the ridge in Table 7, the highest cell density was 1.37E + 06 cells/mL, but this prediction was not very reliable due to a large standard error obtained (144554). Based on the ridge analysis, the glutamine concentrations at high cell densities were found to decreasing, to a level of 1 mM or lower. Glutamine values smaller than 1 mM were thought to

Table 7 Ridge of steepest ascent for *X*1, *X*2, *X*3 independent variables, and estimated response and standard error

X1	*X*2	*X*3	Estimated cell density (10^5)	SE
−0.15	0.07	0.11	10.1	62346
−0.33	0.13	0.18	10.4	61381
−0.52	0.20	0.23	10.7	60421
−0.71	0.26	0.26	11.0	60558
−0.91	0.32	0.28	11.4	63278
−1.10	0.38	0.30	11.8	70025
−1.29	0.43	0.31	12.2	81626
−1.49	0.49	0.33	12.7	98153
−1.68	0.55	0.34	13.2	119266
−1.87	0.60	0.35	13.7	144554

be unreasonable and therefore additional experiments were conducted on the ridge below glutamine values of 1 mM.

To further explore the surface, we used the reduced model from Table 7, and obtained predicted cell densities at constant glutamine concentrations at different coded levels from '0' to '−3', NEAA at '0.5' and various values of EAA. The results are shown in Table 8. The results suggest that cell density increases as EAA (*X*2) increases when glutamine values are low. Figure 4 shows the effect of EAA and NEAA on VCD when glutamine is controlled at coded level '−1'.

EAA values up to a coded level of 14.5 (uncoded value = 12.625 *X*) are unfeasible because of osmotic effects or inhibition of metabolic pathways due to overfeeding the nutrients. According to the above results, however, it appears that with reduced Glutamine levels and concentrations of EAA and NEAA at a 0.5 coded level, large cell densities could possibly be obtained.

Table 8 Effect on EAA, NEAA and VCD when glutamine is controlled at different coded levels

*X*1(level)	*X*2(Level)	*X*3(Level)	VCD
−0.5	4.5	0.5	1.27E + 06
−1	6.5	0.5	1.56E + 06
−1.5	8.5	0.5	1.95E + 06
−2	10.5	0.5	2.43E + 06
−2.5	12.5	0.5	3.01E + 06
−3	14.5	0.5	3.69E + 06

Alternate experiments

To further evaluate the surface we ran some alternate experiments at different levels of glutamine (from coded level '0' to' −3'), keeping EAA constant at coded levels 2 and 4. We expected low cell growth at a glutamine value less than '−1.5 coded level' and no cell growth at zero ('−2' coded level) glutamine concentration. We also expected the EAA coded level of '2' to result in higher cell densities compared to EAA coded level '4,' due to osmotic effects and the inhibition of metabolic pathways from overfeeding. Therefore 14 additional experiments were conducted, four on the ridge, four at different levels of Glutamine keeping EAA at coded level '2,' four at EAA coded level '4,' and two controls (basal medium) as shown in Table 9.

These experiments were conducted under the same conditions as the initial experiments. The starting density of the cultures was 2×10^5 cells/mL, and the cells were allowed to adapt to the medium in four passages. The final viable cell densities were derived as an average of the third and the fourth passage, as shown in Table 9.

Medium-6 had a higher cell density compared to the controls (13 and 14), but the last passage of medium 3, 9, and 5 were nearly equal to the control medium, as shown in Fig. 5. Media which had results equivalent to, or better than medium-0, were carried out for one more passage (up to 8 days), to validate the data. The results are shown in Fig. 6. From passage 5, the viable cell density attained in medium 6 after 5 days of culture was about 1.6 times higher than the control.

Replicate experiments

To validate the above results, the cells in the control medium were taken out of a frozen state, and the experiment was repeated three times with control medium and medium-6. Cells were allowed to go for four passages and the final viable cell densities were taken as an average of passage 3 and passage 4. The results are presented in Fig. 7.

In medium-6, a viable cell density of 1.45×10^6 cells/mL was attained, which was found to be 1.4 times higher than for the control medium and within two standard errors of 1.23E + 06 from the original run of medium 6 (standard error = 215,928 cells/mL

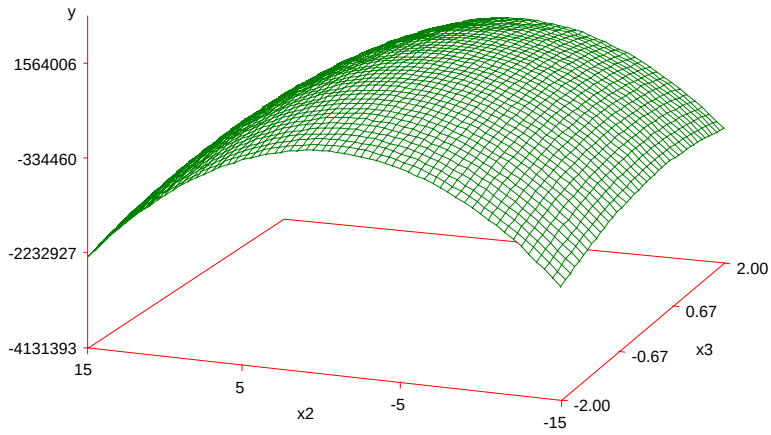

Fig. 4 Response surface plot showing the effect of EAA (*X*2), NEAA (*X*3) and their mutual effect on the *Y* (viable cell density), when glutamine (*X*1) is controlled at −1 coded level

Table 9 Alternate experiments carried out on ridge and glutamine controlled at different coded levels, keeping EAA constant at coded levels 2 and 4

Medium	*X*1	*X*2	*X*3	VCD (105)	SD
1	−0.52	0.196	0.225	8.13	203490
2	−1.1	0.375	0.299	8.81	179368
3	−1.487	0.491	0.329	8.61	60052
4	−1.874	0.604	0.352	8.01	152664
5	0	2	0.5	9.35	42230
6	−1	2	0.5	1.23	215928
7	−2	2	0.5	7.23	277564
8	−3	2	0.5	1.79	45162
9	0	4	0.5	7.93	102429
10	−1	4	0.5	4.49	152555
11	−2	4	0.5	5.7	144684
12	−3	4	0.5	2	52915
13	0	0	0	9.15	134722
14	0	0	0	9.48	121484

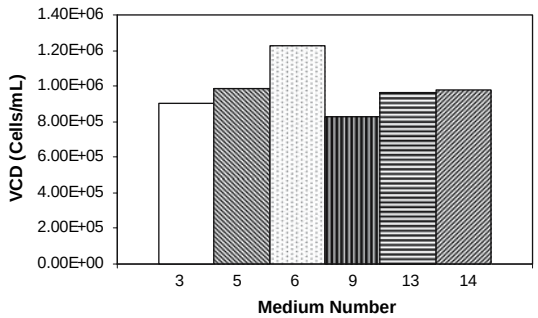

Fig. 5 Viable cell density versus medium number tested. The plot shows the viable density obtained in the last passage (passage 4) of alternate experiments undertaken. Details on media are listed in Table 9

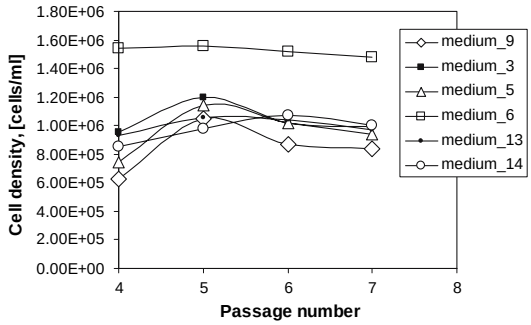

Fig. 6 Results of the alternate experiments conducted to further explore the surface

for medium 6). After four passages, if the cells were allowed to grow to passages of 5–7 the viable cell density obtained in medium-6 increased to 1.6×10^6 cells/ml. The viable cell density in the control experiment in these passages (passages 5–7) was between 9.2×10^5 cells/mL and 1.1×10^6 cells/mL, with in the standard deviation of 128,103 cells/mL (data not shown).

Assuming the specific antibody production (mg protein per cell per day) depends on the cell density, we expect to see an increase in the specific antibody production with an increase in the cell density. As detailed in the methods section, transformed cells were cultured in control and the optimized medium, medium-6, and the antibody titer was estimated in the supernatant, the specific antibody production was determined. Standard deviation was calculated in these media from triplicate experiments. The results are shown in Fig. 8. The antibody titer was determined for all the initial medium (∼50) experiments, and the results were analyzed using SAS/

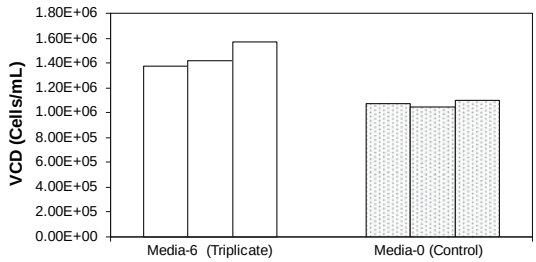

Fig. 7 Results of the triplicate experiments of Medium-6 and the Control Medium

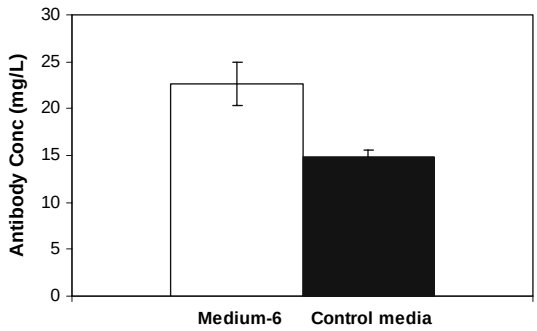

Fig. 8 Antibody production in Medium-6 and the Control Medium

STAT procedures. We observed the same trend for antibody production as well, where the stationary point is a saddle point, and the ridge values for the glutamine were moving in the negative direction and in positive direction for EAA and NEAA, shown in Table 10. The R^2 value for the model was 0.75 which shows adequacy of the model in explaining the effect of the variables on the response which is antibody production. Thus the antibody titer in medium-6 was estimated to be 1.6 times higher than the control medium and the composition of the variables in medium-6, that was found to be optimal in our present study, is listed is Table 11.

Conclusions

The increase in the Viable cell density (Cells/mL), and the production of the antibody against BoNT-A was accomplished by using Box-Wilson's Central Composite Design.

In the optimal media, a 1.4 times higher in the viable cell density and a 1.6 times higher found antibody titer was obtained. Lower levels of glutamine and higher levels of EAA are preferred. From the results presented here, we expect that one of the amino acids is replacing the role of amino acid-glutamine, and may be acting as limiting nutrient. Thus in our future and ongoing efforts, we will undertake amino acid analysis, to identify the limiting amino acid. We speculate that the addition of the limiting amino acid, albeit separately, separately, a decrease in the concentration of the total amino acid can be engineered, thus make the medium more economical. We also anticipate increased cell densities and higher antibody titers.

Table 10 Ridge of steepest ascent of the reduced model for getting maximum antibody production with independent variables $X1$, $X2$, $X3$. $X4$ and $X5$ are kept constant at their zero−level

$X1$	$X2$	$X3$	Estimated response (mg/L)
0	0	0	12.73
−0.172	−0.01	0.101	13.46
−0.34	−0.001	0.2	14.21
−0.522	0.03	0.293	15
−0.698	0.08	0.38	15.82
−0.874	0.149	0.461	16.69
−1.049	0.226	0.536	17.61
−1.22	0.31	0.607	18.58
−1.39	0.4	0.673	19.6
−1.56	0.496	0.736	20.6
−1.73	0.595	0.796	21.8

Table 11 Concentrations of the five variables of the optimal medium

Variables	Concentration
Glutamine	2.5 mM
EAA	3.25 × (1.67 g/L)
NEAA	2.125 × (0.168 g/L)
ITS	1 × (0.22 g/L)
Lipids	0.7 × (0.71 g/L)

Total concentration of all amino acids in EAA and NEAA are given in g/L. Concentrations of all other components are as given in Table 2

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